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# Effects of simulated microgravity on arterial nitric oxide synthase and nitrate and nitrite content

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**Ma, Jin, Chadi I. Kahwaji, Zhenmin Ni, Nosratola D. Vaziri, and Ralph E. Purdy.** Effects of simulated microgravity on arterial nitric oxide synthase and nitrate and nitrite content. *J Appl Physiol* 94: 83–92, 2003. First published September 20, 2002; 10.1152/japphysiol.00294.2002.—The aim of the present work was to investigate the alterations in nitric oxide synthase (NOS) expression and nitrate and nitrite (NOx) content of different arteries from simulated microgravity rats. Male Wistar rats were randomly assigned to either a control group or simulated microgravity group. For simulating microgravity, animals were subjected to hindlimb unweighting (HU) for 20 days. Different arterial tissues were removed for determination of NOS expression and NOx. Western blotting was used to measure endothelial NOS (eNOS) and inducible NOS (iNOS) protein content. Total concentrations of NOx, stable metabolites of nitric oxide, were determined by the chemiluminescence method. Compared with controls, isolated vessels from simulated microgravity rats showed a significant increase in both eNOS and iNOS expression in carotid arteries and thoracic aorta and a significant decrease in eNOS and iNOS expression of mesenteric arteries. The eNOS and iNOS content of cerebral arteries, as well as that of femoral arteries, showed no differences between the two groups. Concerning NOx, vessels from HU rats showed an increase in cerebral arteries, a decrease in mesenteric arteries, and no change in carotid artery, femoral artery and thoracic aorta. These data indicated that there were differential alterations in NOS expression and NOx of different arteries after hindlimb unweighting. We suggest that these changes might represent both localized adaptations to differential body fluid redistribution and other factors independent of hemodynamic shifts during simulated microgravity.

rat; artery; hindlimb unweighting

ADAPTATION TO MICROGRAVITY during spaceflight can cause astronauts to experience orthostatic intolerance on return to the gravity of Earth. Symptoms experienced during standing can include increased heart rate, orthostatic hypotension, and frank syncope. These alterations in orthostatic stability can adversely affect the performance of physical work (41, 56). Even after several decades of study, the basic mechanisms underlying orthostatic intolerance are not fully under-

stood. It is well known that no single factor can account for postflight orthostatic intolerance. However, multiple mechanisms, including hypovolemia, cardiovascular structural changes, and alterations in central integration, baroreceptor function, and neurohumoral regulation, may operate together to cause postflight orthostatic intolerance on return to the gravitational environment (1, 41, 56, 62, 63).

The results of human and animal studies in both real and simulated microgravity strongly suggest that there are multiple changes in structure and function of arterial vasculature that could contribute to postflight orthostatic intolerance (7–10, 18, 27, 28, 45, 57, 62). In human studies, Mulvagh et al. (38) found that the total vascular resistance index of 24 crew members was significantly decreased immediately after short-term spaceflight. Buckey et al. (3) reported that only 5 of the 14 crew members could finish a 10-min stand test after 9–14 days of spaceflight. Analysis of the data showed that the postural vasoconstrictor response was significantly greater among the finishers. Furthermore, evidence suggests that microgravity-induced changes in the cerebral vasculature may cause decreased brain blood flow and thus, play an important role in the genesis of postflight orthostatic intolerance (2, 13, 65).

In animal studies, data from different groups have shown that simulated microgravity causes differential functional and structural changes among arteries. Both decreased arterial vasoconstrictor responses and decreased myogenic tone development are likely to contribute to a diminished ability to raise peripheral resistance in animals subjected to simulated microgravity (7, 25). The increased myogenic tone reported by Geary et al. (15) and the enhanced vasoconstrictor responsiveness reported by Zhang et al. (64) in cerebral arteries after simulated microgravity support the view that increased cerebrovascular resistance may lead to decreased brain blood flow in astronauts exposed to orthostatic challenge.

Among the many factors influencing arterial structure and function, the release of vasoactive substances from the endothelium is now recognized as a major

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mechanism regulating vascular tone and local blood flow (16, 37). In turn, other studies have demonstrated that differential arterial hemodynamics among vascular beds leads to differential alterations of endothelial function (22–24). This can be illustrated by comparing simulated microgravity studies of rat carotid arteries vs. soleus feed arteries. Sangha et al. (48) provided functional evidence that either the activity or expression of endothelial nitric oxide synthase (eNOS) is increased in the carotid artery by hindlimb unweighting (HU). In contrast, Jasperse et al. (20) showed that eNOS expression is decreased by HU in the soleus feed arteries. Shear stress is a powerful stimulus for the modulation of eNOS expression (35, 39, 60) and is likely to have been responsible for the differential changes in the eNOS expression in these blood vessels. In vivo, shear stress was not determined in the carotid artery but was likely to be increased by HU on the following grounds. HU causes an ~20-mmHg increase in pressure in the ascending aorta (59), and a parallel increase is likely to occur to the carotid arteries. In contrast, Delp et al. (9) showed that HU causes a marked decrease in shear stress in the soleus feed artery, and McCurdy et al. (32) argued that this decrease accounted for the reduced eNOS expression in this vessel.

In a previous study (48), our laboratory found functional evidence that inducible nitric oxide synthase (iNOS) activity was increased by HU in the femoral artery and accounted, in part, for the HU-induced hyporesponsiveness of this vessel to norepinephrine (NE). In addition, our laboratory found in another study (53) that HU increased iNOS expression in many cardiovascular and noncardiovascular tissues. Moreover, iNOS blockade in vivo was found to cause a greater pressor response in HU than in control rats (53). The results of these two studies led us to propose that simulated microgravity may cause a generalized upregulation of nitric oxide synthase (NOS). As stated above, upregulation of eNOS in arteries of the upper body is likely to be dependent on increased shear stress. On the other hand, the mechanisms regulating iNOS expression in simulated microgravity are unknown.

The present study is based on the convergence of two ideas. The first is the possibility that HU increases NOS expression, causing a chronic elevation of nitric oxide (NO) in the vasculature. In turn, this could account, in part, for the HU-induced vascular hyporesponsiveness. The second is the hypothesis, expressed by Zhang et al. (61, 63), that the microgravity-induced nonuniform redistributions of transmural pressures and flows are responsible for the vascular effects of HU. Zhang (61) reviewed the evidence that the hemodynamic effects of microgravity initiate differential, functional, and structural adaptations among arteries. In the present study, eNOS and iNOS were measured to assess possible HU-induced changes in NOS expression. Vascular tissue content of nitrate and nitrite (NOx), the stable metabolites of NO, were measured to

determine whether HU causes changes in tissue NO content.

These parameters were measured in the following blood vessels in which HU produces differential in vivo hemodynamic effects: thoracic aorta and carotid artery, which experience increased pressure and/or flow (50, 59); femoral artery, which experiences decreased pressure and/or flow (47); and the superior mesenteric arcade in which pressure is either unchanged (33) or minimally increased (50). The cerebrovasculature was also assessed. Numerous studies suggest that simulated microgravity increases blood flow in this vascular bed (12, 21, 50). On the other hand, Wilkerson et al. (58) reported recently that blood flow is decreased by HU in the rat cerebrovasculature. The goal of the present study was to detect HU-induced changes in NOS expression and tissue content of NOx. In turn, this would allow an assessment of the possible contribution of changes in these parameters to the previously reported vasoconstrictor effects of HU in the blood vessels studied. In addition, it might provide insight into the possible contribution of the known hemodynamic effects of HU to the HU-induced changes in NOS and NOx.

## MATERIALS AND METHODS

**Animal model.** The methods employed in this study were approved by The Institutional Animal Care And Use Committee Of The University Of California, Irvine, and were in accordance with the guidelines on the care and use of animals required by The American Physiological Society.

The rat HU model, described in detail previously (4, 53, 64), was used to simulate cardiovascular-deconditioning effect of microgravity. Male Wistar rats weighing 250–300 g (Simonsen Laboratories, Gilroy, CA) were obtained and caged individually in a room maintained at 23°C in a 12:12-h light-dark cycle. Water and standard laboratory chow were provided ad libitum. After 1 wk, the rats were randomly assigned to a HU or a control group. For simulating microgravity, HU animals were subjected to tail suspension for 20 days. HU was achieved by the use of a tail harness that partially elevated the hindlimbs above the floor of the cage. The rat tail was cleaned and dried, and a coat of benzoin tincture (Graeco, Hauppauge, NY) was applied and then allowed to air dry. A thin adhesive tape strip (Zimmer, Dover, OH) with a width sufficient to cover one-fourth of the tail girth was adhered laterally along the two sides of the proximal two-thirds of the tail. These longitudinal strips were then secured to the tail by three, 1-cm-wide tape strips (Beiersdorf-Jobst, Rutherford College, NC) wrapped circumferentially at three sites along the length of the tail. The rats were suspended via a tether connecting the tail harness to a horizontal tube at the top of the cage. The upper end of the tether included a small pulley that rolled freely along the length of the horizontal tube at the top of the cage. The cage floor was made of parallel plastic circular tubes 1 cm in diameter, with 0.8-cm spaces between tubes. These designs allowed the rats to move freely around the cage and prevented them from grasping the cage floor to pull against the tail harness. The animals were maintained in ~35° head-down tilt, with the hindlimbs elevated ~0.5 cm above the floor when fully extended.

**Tissue harvest.** After 20 days, rats were euthanized by exposure to 100% CO<sub>2</sub> for 90 s to induce deep anesthesia (17)

and subsequent opening of the chest cavity. The cerebral, carotid, mesenteric, and femoral arteries and the thoracic aorta were removed. The cerebral arteries included the anterior, middle, and posterior cerebral arteries, the circle of Willis, and the basilar artery. The mesenteric arteries included all branches of the superior mesenteric artery from the first to the fifth order. All arteries were cleaned under a dissecting microscope to remove fat and connective tissues.

**NOS expression.** eNOS and iNOS protein content were measured by Western blotting described in detail previously (52, 54, 55). Briefly, arteries were homogenized in 10 mM HEPES lysis buffer, pH 7.4, containing 320 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10  $\mu$ g/ml leupeptin, and 2  $\mu$ g/ml aprotinin at 0–4°C with the aid of a tissue grinder fitted with a motor-driven ground glass pestle. Homogenates were centrifuged at 12,000 *g* for 5 min at 4°C to remove nuclear fragments and tissue debris without precipitating plasma membrane fragments. The supernatant was used for determination of NOS isoform proteins. The total protein concentration of supernatant was determined by using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Arterial tissue supernatant (40  $\mu$ g of protein) was size-fractionated on 8% Tris-glycine gel (Novex, San Diego, CA) at 120 V for 4 h by using a Bio-Rad power system. In preliminary experiments, we had found that the given protein concentrations were within the linear range of detection for our Western blot method. After gel electrophoresis, proteins were transferred from gel onto Hybond-enhanced chemiluminescence (ECL) membrane (Amersham Life Science, Arlington Heights, IL) at 500 mA for 90 min by using the Novex transfer system. The membrane was blocked for 1 h at room temperature with blocking buffer (10 mM Tris-hydrochloride, pH 7.4, 100 mM NaCl, 0.1% Tween 20, and 10% nonfat milk powder). Then the membrane was incubated overnight at 4°C with primary antibody against eNOS or iNOS (1:1,500; Transduction Laboratories, San Diego, CA). The membrane was then washed for 30 min in shaking bath, changing the washing buffer (blocking buffer without nonfat milk powder) every 5 min. Subsequently, the membrane was incubated for 1 h in washing buffer with secondary antibody (1:2,000; horseradish peroxidase-conjugated anti-mouse IgG) at room temperature. The washes were repeated for 30 min, then either specific eNOS or iNOS was detected by enhanced chemiluminescence (ECL) method by using ECL reagent (Amersham Life Science) and evaluated by a laser densitometer (model PD1211, Molecular Dynamics, Sunnyvale, CA). In all instances, the membranes were stained with Ponceau stain, which verified the uniformity of protein load and transfer efficiency across the test samples.

**NOx content measurement.** Arteries were homogenized in 10 mM HEPES buffer, pH 7.4, containing 1 mM EDTA, 1 mM DTT, 10  $\mu$ g/ml leupeptin, and 2  $\mu$ g/ml aprotinin at 0–4°C with the aid of a Polytron homogenizer. Homogenates were centrifuged at 12,000 *g* for 5 min at 4°C and the supernatant was used for determination of NOx content by the chemiluminescence method using the purge system of a Sievers Instruments model 270B NO analyzer (NOA, Sievers Instruments, Boulder, CO) (53). Briefly, 5 ml of filtered saturated solution of VCl<sub>3</sub> in 1 M HCl were added to the purge vessel and purged with nitrogen gas for 10 min before use. The gas flow rate into the chemiluminescence detector was controlled to yield a cell pressure of 7 Torr. Samples were injected into the purge vessel to react with the VCl<sub>3</sub>-HCl reagent at 95°C, which converted NOx to NO. The NO was stripped from the reaction chamber and detected by ozone-induced chemiluminescence in the chemiluminescence detector. The signal generated was recorded and processed by a Hewlett Packard

model 3390 Integrator. Standard curves were constructed by using various concentrations of NO<sub>3</sub><sup>-</sup> and relating the luminescence produced to the given concentrations. The amount of NOx in each sample was determined by interpolation on the standard curve. The total protein concentration of sample was determined by using a Bio-Rad protein assay kit (Bio-Rad Laboratories).

**Statistical analysis.** Values are presented as means  $\pm$  SE, and unpaired *t*-tests were made between groups. *P* < 0.05 was required for significance.

## RESULTS

The experiments carried out in this study were designed to measure the effects of HU on eNOS, iNOS, and NOx in vessels from five anatomically distinct regions of the body. The results obtained in the cerebrovasculature are shown in Fig. 1. HU treatment had no effect on either eNOS or iNOS expression. In contrast, NOx was significantly increased. This suggests that NOS activity was increased by HU in the cerebrovasculature.

In the carotid artery, both eNOS and iNOS were increased by HU. NOx exhibited a nonsignificant trend toward increase (see Fig. 2). The thoracic aorta exhibited a pattern identical to that of the carotid artery. As shown in Fig. 3, HU increased both eNOS and iNOS expression, with only a nonsignificant trend toward an increase in NOx in thoracic aorta.

HU caused a marked decrease in both eNOS and iNOS expression in the mesenteric vasculature (Fig. 4). This was accompanied by a significant decrease in NOx.

The results obtained in the femoral artery are shown in Fig. 5. HU has no effect on eNOS, iNOS, or NOx. This was surprising in light of our laboratory's previous study providing functional evidence for increased iNOS activity in the femoral artery (48).

## DISCUSSION

The goal of the present study was to determine the effects of HU on eNOS and iNOS expression and on NOx content in several arteries. The arteries selected for study have been shown to experience differential hemodynamic effects *in vivo* during HU treatment. Thus comparison of these vessels could provide insight into whether there is a general pattern relating NOS expression and hemodynamic events. In addition, it was of interest to determine the extent to which changes in NOS and NOx found in the present study could account for the previously reported contractile effects of HU in these vessels.

HU had the same effects on both eNOS and iNOS in all vessels studied. For example, both isoforms of NOS were increased in thoracic aorta and carotid artery, decreased in the mesenteric vasculature and unchanged in both the femoral artery and the cerebrovasculature. This could imply that the various factors regulating NOS expression affect eNOS and iNOS in a parallel manner. However, the mechanisms regulating NOS expression are likely to be more complex than this, as discussed below.

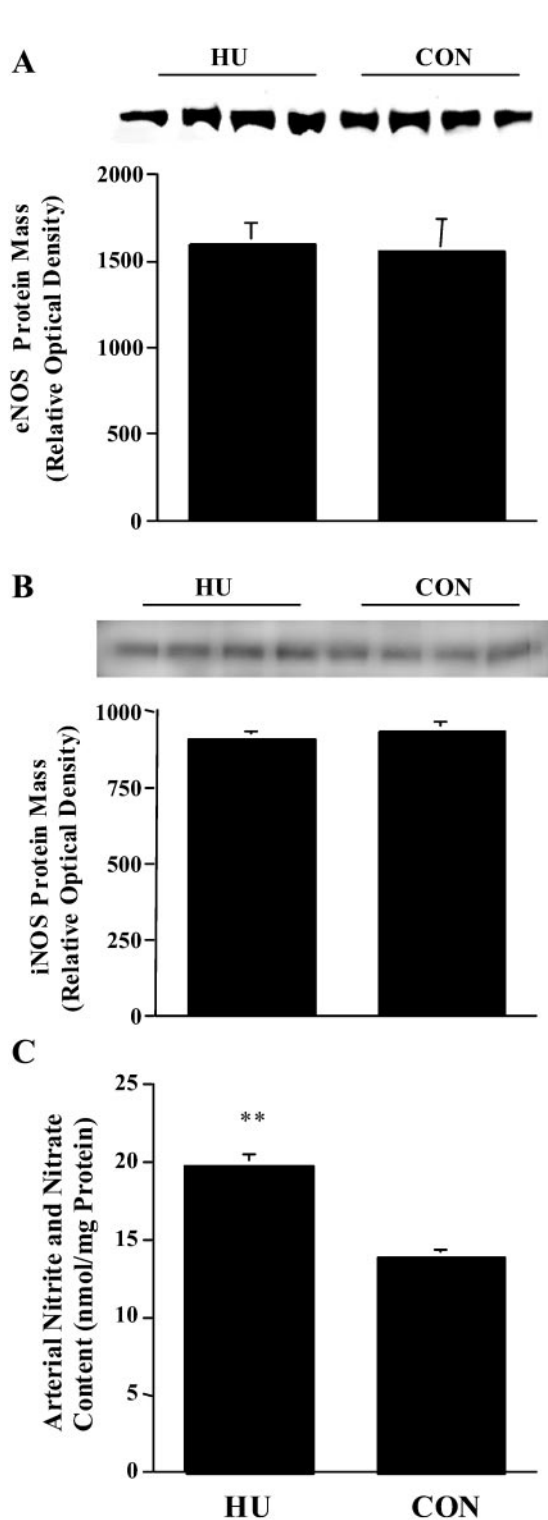


Fig. 1. Nitric oxide synthase protein expression and nitrite and nitrate in cerebral arteries isolated from 20-day hindlimb-unweighting (HU) and control (Con) rats. **A**: endothelial nitric oxide synthase (eNOS;  $n = 4$  in each group, each sample based on tissue pooled from 6 animals). **B**: inducible nitric oxide synthase (iNOS;  $n = 4$  in each group, each sample based on tissue pooled from 6 animals). **C**: nitrite and nitrate ( $n = 6$  in each group, each sample based on tissue pooled from 6 animals). Values are means  $\pm$  SE.  $^{**}P < 0.01$  vs. Con.

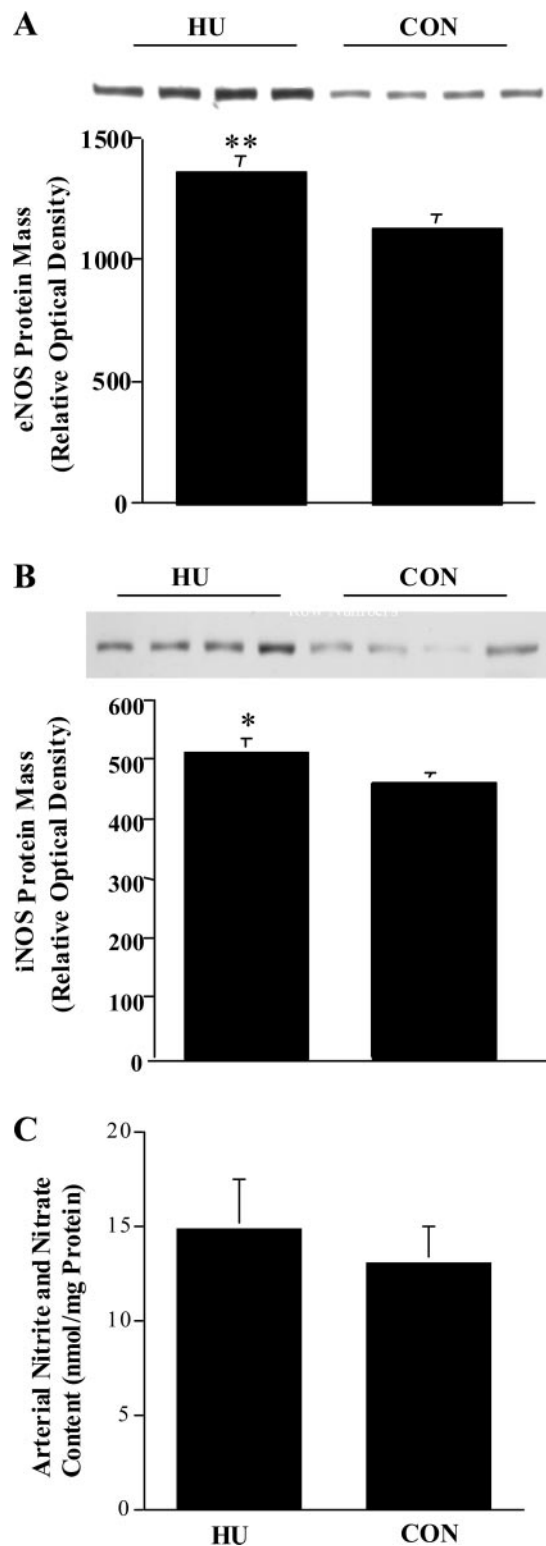


Fig. 2. Nitric oxide synthase protein expression and nitrite and nitrate in carotid arteries isolated from HU and Con rats. **A**: eNOS ( $n = 4$  in each group, each sample based on tissue pooled from 3 animals). **B**: iNOS ( $n = 4$  in each group, each sample based on tissue pooled from 3 animals). **C**: nitrite and nitrate ( $n = 9$  in each group, each sample based on tissue pooled from 3 animals). Values are means  $\pm$  SE.  $^{*}P < 0.05$ ,  $^{**}P < 0.01$  vs. Con.

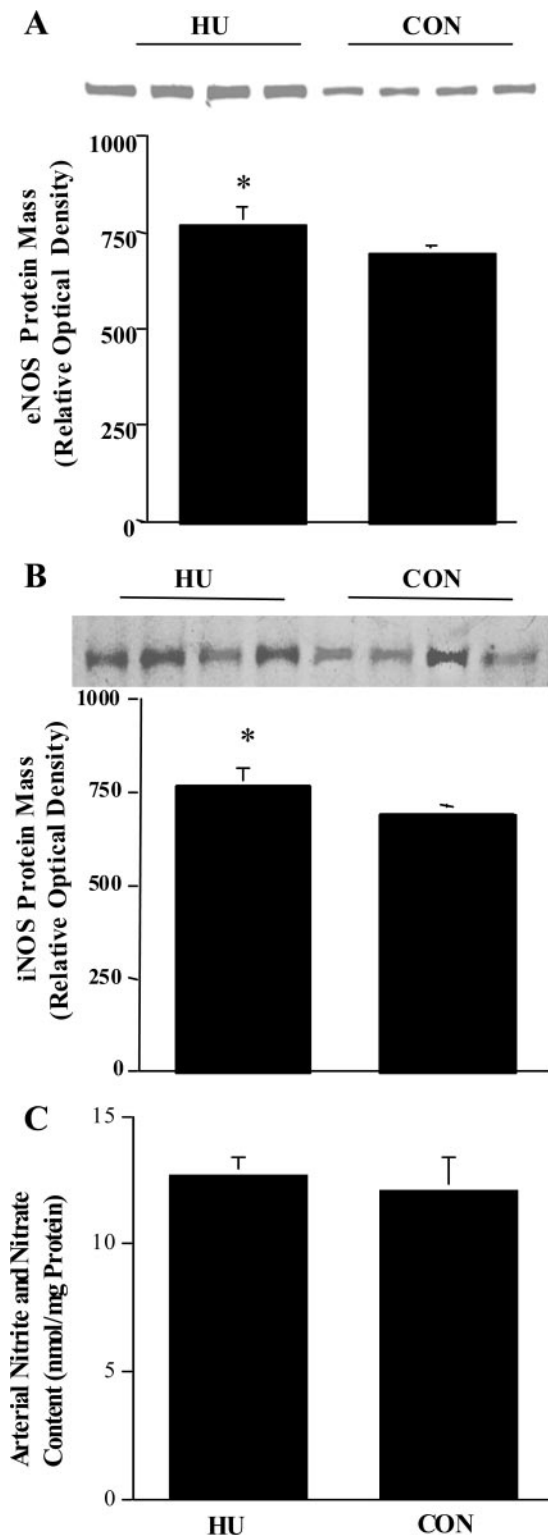


Fig. 3. Nitric oxide synthase protein expression and nitrite and nitrate in thoracic aortas isolated from HU and Con rats. A: eNOS ( $n = 4$  in each group). B: iNOS ( $n = 4$  in each group). C: nitrite and nitrate ( $n = 8$  in each group). Values are means  $\pm$  SE. \* $P < 0.05$  vs. Con.

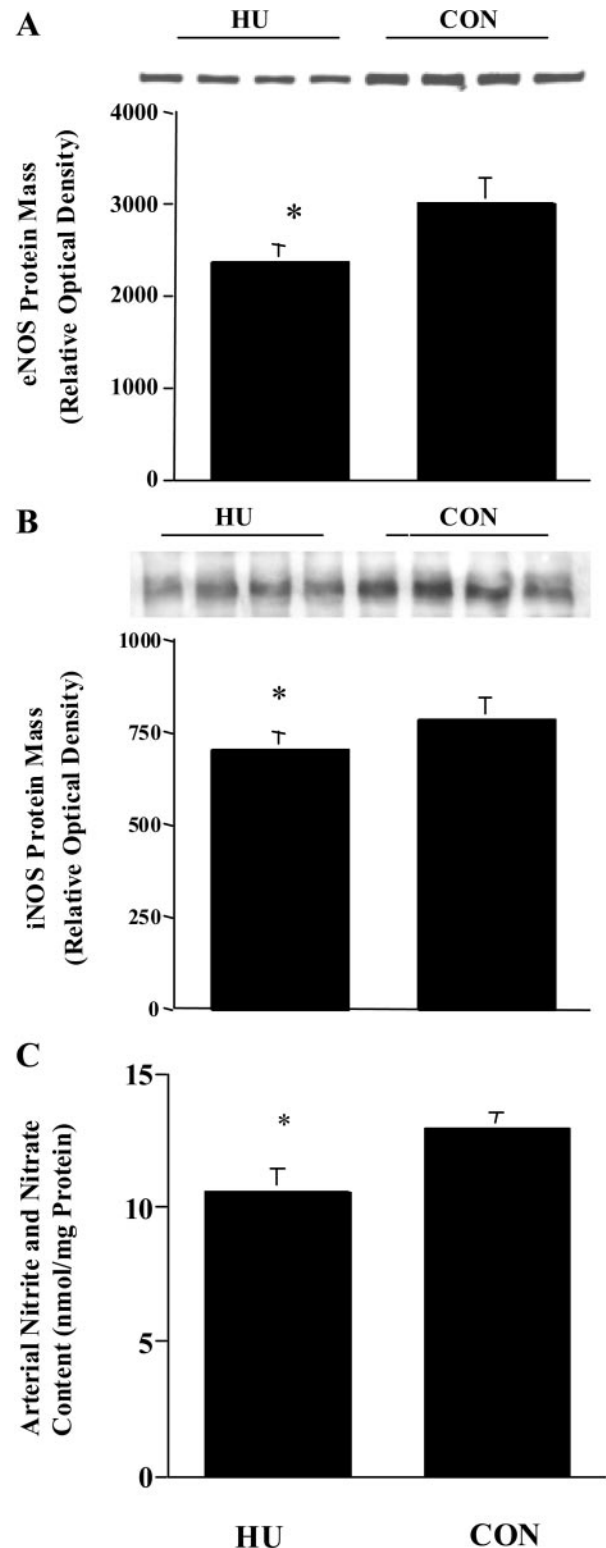


Fig. 4. Nitric oxide synthase protein expression and nitrite and nitrate in mesenteric arteries isolated from HU and Con rats. A: eNOS ( $n = 4$  in each group). B: iNOS ( $n = 4$  in each group). C: nitrite and nitrate ( $n = 10$  in each group). Values are means  $\pm$  SE. \* $P < 0.05$  vs. Con.

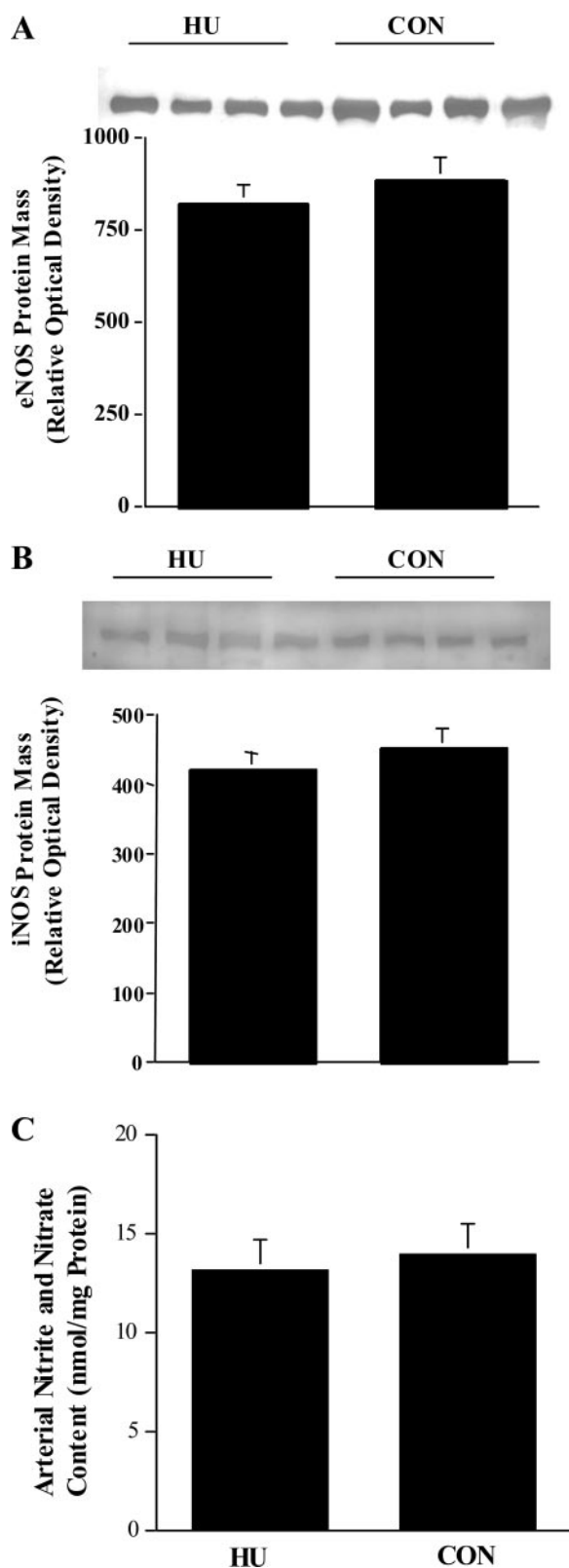


Fig. 5. Nitric oxide synthase protein expression and nitrite and nitrate in femoral arteries isolated from HU and Con rats. A: eNOS ( $n = 4$  in each group, each sample based on tissue pooled from 3 animals). B: iNOS ( $n = 4$  in each group, each sample based on tissue pooled from 3 animals). C: nitrite and nitrate ( $n = 10$  in each group, each sample based on tissue pooled from 3 animals). Values are means  $\pm$  SE.

NOx are stable metabolites of NO. Thus the presence of NOx in an artery is a reflection of NO synthesis and release within that blood vessel. The magnitude of the tissue NOx is dependent on both the amount of NOS enzyme present and the activity of the enzyme. If activity is not different between tissues, then NOx reflects the amount of enzyme present.

NOx levels tended to change in the same direction as NOS in most of the blood vessels studied. For example, eNOS and iNOS were increased in both carotid artery and aorta and were decreased in mesenteric artery. Accompanying the modest increases in NOS in carotid artery and aorta were nonsignificant increases in NOx. Accompanying the more marked decreases in NOS in the mesenteric artery was a significant decrease in NOx. There were no HU-induced changes in either NOS or NOx in the femoral artery. These comparisons suggest that NOx reflected the synthesis and release of NO in the four vessels discussed, but with low sensitivity.

The effects of HU on NOS and NOx in the cerebrovasculature differed from those in the four vessels discussed above. HU had no effect on cerebrovascular eNOS or iNOS, but it produced a significant increase in NOx. The mechanisms underlying this increase in NOx in the face of unchanged NOS are unknown. However, there are at least two possible explanations. First, although cerebrovascular eNOS and iNOS expressions were unchanged, it is possible that the activity of either or both of these enzymes was increased by HU. Second, it is possible that neuronal NOS (nNOS) expression was increased in the cerebrovasculature by HU and that this isoform was responsible for the HU-induced increase in NOx. The larger pial vessels, such as those assessed in the present study, possess nNOS in both the endothelium and the perivascular nerves (51). In support of this possibility, our laboratory found previously that HU increased nNOS in both brain and kidney (53).

The present finding that NOx was increased by HU in the cerebrovasculature may have functional implications. We studied the rat middle cerebral artery in a previous study (15) and found that HU markedly increased myogenic tone in this vessel. Blockade of NOS with  $N^G$ -L-nitro-arginine methyl ester had little further effect on the myogenic tone of the HU middle cerebral artery, but markedly increased myogenic tone in the control vessel. After NOS blockade, the levels of myogenic tone in the control and HU middle cerebral arteries were not significantly different. It was concluded that HU increased myogenic tone in the cerebrovasculature, in a large part, by attenuating the contribution of NO-mediated vasodilation.

A more recent study from our laboratory (14) lends support to this conclusion. With the use of endothelium-denuded middle cerebral arteries, it was found that HU markedly attenuated the vasodilator response to the NO donor sodium nitroprusside. Such desensitization in isolated blood vessels has been shown to be associated with prior elevated exposure to endogenous NO (6, 43). Thus the present finding that HU increased

NO<sub>x</sub> is consistent with the possibility that HU increased the concentration of NO within the cerebrovasculature, leading to the desensitization to sodium nitroprusside observed by Geary et al. (14). Future studies are required to determine the source of the elevated NO metabolites found in the HU cerebrovasculature.

The cerebrovasculature was included in the present study because cerebral blood flow was believed to be increased in humans subjected to simulated microgravity (12, 21) and was shown to be increased in the brains of HU rats (50). However, Wilkerson (58) has shown recently that blood flow is decreased acutely, and for up to 28 days, in all brain regions of the HU rat. Thus, because of these conflicting findings on brain blood flow in the HU rats, it is not possible currently to relate the present findings on NOS and NO<sub>x</sub> to HU-induced hemodynamic changes in the cerebrovasculature.

It was hypothesized in the present study that eNOS expression would be increased by HU in the carotid artery and thoracic aorta. In the carotid artery, this was based on the finding that brain blood flow was increased by HU (50). This implies that HU may also increase blood flow in the carotid artery, the main distributing artery feeding the brain. Blood flow has not been measured in the thoracic aorta. However, HU was shown to increase pressure in the thoracic aorta (59), raising the possibility that flow could also be increased by HU in this vessel. Consistent with these possibilities, eNOS expression was increased in both vessels. In an earlier study, our laboratory obtained functional evidence that the HU-induced reduction in the contractile capacity of the carotid artery was mediated, in part, by an endothelial effect (48). Namely, 1) endothelium removal partially restored the impaired NE-induced contraction in HU carotid artery and 2) HU carotid arteries were 10-fold more sensitive than control to the endothelium-dependent vasodilator effects of acetylcholine. The present finding that eNOS expression was increased in HU carotid artery provides a mechanistic explanation for our laboratory's earlier observations (48).

HU increased iNOS expression in thoracic aorta, in agreement with our laboratory's earlier study (53). HU also increased iNOS expression in the carotid artery. This agrees with another study from our laboratory that provided functional evidence in support of an HU-induced increase in iNOS (48). In that study (48), exposure to the NOS substrate, L-arginine, relaxed precontracted HU carotid artery, and that relaxation was reversed by selective iNOS blockade. In addition, iNOS blockade partially restored the impaired NE-induced contraction in HU carotid artery. Thus the increased iNOS expression found in the present study supports the findings of our laboratory's earlier study and suggests that the HU-induced elevation of iNOS may contribute to the HU-mediated hyporesponsiveness of the carotid artery.

HU had no effect on eNOS expression in the femoral artery. This result was unexpected in light of the fact

that HU reduces flow and, therefore, shear stress in this blood vessel. For example, Roer and Dillaman (47) reported that HU decreased blood flow in the femoral artery by ~40% for 7 days. Moreover, Collieran et al. (5) found that HU reduced blood flow in hindlimb muscle and bone for up to 28 days. Thus it is likely that the femoral artery blood flow was reduced by HU for the 20-day HU treatment used in the present study.

Because shear stress is directly related to blood flow, it was reasoned in the present study that shear stress would be reduced in the femoral artery, reflecting the HU-induced reduction of blood flow in this vessel (47). As a consequence, it was the hypothesis of the present study that eNOS expression would be reduced in HU, compared with control femoral artery. Although there was a trend toward reduction of eNOS in HU, this trend did not achieve statistical significance (Fig. 5A).

Studies of shear stress (9) and eNOS expression (20) in the soleus feed arteries provide a possible explanation for the present results in femoral artery. HU reduced blood flow in the soleus feed arteries for at least 14 days (9). Shear stress was markedly reduced by HU initially but returned to pretreatment levels by 14 days of HU (9). The factor responsible for this reversal of shear stress was the reduction in soleus feed artery internal diameter. The time course of this structural remodeling of the soleus feed artery is unknown. However, it is likely to have required at least several days. This is based on the finding by Jasperse et al. (20) that eNOS expression was reduced at 14 days of HU. Thus the reduced shear stress must have been of sufficient duration to downregulate eNOS expression and to prevent its return to the control level during the 14 days of HU.

On the basis of the above discussion, the lack of effect of HU on eNOS expression in femoral artery might be understood in terms of the competing effects of blood flow and vessel diameter on shear stress. In accordance with the studies by Roer and Dillaman (47) and Collieran et al. (5), blood flow was likely to have been reduced throughout the 20 days of HU used in the present study. Regarding artery diameter, Mao et al. (30) found that femoral artery diameter was reduced at 28 days of HU. Although the time course of structural remodeling of the femoral artery is unknown, it could have followed that of the soleus feed artery; i.e., the femoral artery diameter could have been reduced at 14 days, thereby opposing the effects of the reduced blood flow on shear stress. eNOS expression could well have been downregulated by 14 days of HU in the femoral artery but undetected in the present study because recovery to control levels could have occurred at 20 days of HU, when tissues were isolated for analysis. Future studies are needed to explore the time-course of possible changes in eNOS in the femoral artery.

The expression of iNOS was unchanged by HU in femoral artery. This is in contrast to our laboratory's earlier study (48) in which evidence was obtained for a marked HU-induced increase in iNOS function. In that study, two experimental protocols were used to test for iNOS function. In one, femoral artery rings were pre-

contracted with phenylephrine, and the magnitude of vasorelaxation to L-arginine was measured. The involvement of iNOS was assessed by using the selective iNOS inhibitor, aminoguanidine. HU femoral arteries exhibited a markedly greater aminoguanidine-reversible vasorelaxation than control arteries. In the second protocol, NE concentration-response curves were obtained in femoral artery rings in both the presence and absence of exogenously added L-arginine. The contraction to norepinephrine was reduced in HU, compared with control arteries. Blockade of iNOS had no effect in control arteries, but it completely restored the contraction of the HU arteries to the control level in the presence of L-arginine and caused a partial restoration of contraction in the absence of exogenous L-arginine.

The finding in our laboratory's previous study (48) that iNOS function was increased in femoral artery could be explained in two ways. HU could have increased either the expression or the activity of iNOS. The present study was designed to differentiate between these possibilities. However, HU had no effect on iNOS expression in the femoral artery. Moreover, HU had no effect on NOx content. NOx reflects tissue content of NO and was used in the present study as an indirect measure of NOS activity. Therefore, the present findings suggest that HU had no effect on either iNOS expression or activity in the femoral artery. Thus the present results disagree with the functional findings of our laboratory's earlier study (48). Presently, we can offer no explanation for the differences between these two studies. Future experiments are required to resolve this discrepancy.

HU reduced the expression of both eNOS and iNOS in the mesenteric arterial vasculature. Consistent with this, HU also caused a significant reduction in NOx in this vascular bed. The mechanisms underlying these HU-induced changes are unknown. Moreover, it is unlikely that these changes in NOS and NOx can be attributed to the hemodynamic effects of HU. HU has been shown to have either no effect on (33), or to modestly increase (50), blood flow in the mesenteric vasculature.

It also appears that the present findings cannot account for the previously reported functional effects of HU in the mesenteric vasculature. The HU-induced reductions in NOS and NOx reported in the present study represent a reduction in NO-mediated vasodilatory mechanisms. In turn, this would be expected to enhance vasoconstriction. However, HU has been shown to decrease both agonist-induced vasoconstriction (27) and myogenic tone (25) in mesenteric vasculature. Clearly, other mechanisms, independent of NO, are responsible for these functional effects of HU in the mesenteric vascular bed.

It is of interest to compare the effects of HU on NOS observed in the present study with the previously reported hemodynamic effects of HU in the vessels studied. HU changed eNOS and iNOS in parallel in the different blood vessels. Because the mechanisms underlying the effects of either HU or hemodynamic change on iNOS are unknown, this comparison can

only address a simple question. Is there a general pattern that links NOS expression with hemodynamic change? The answer appears to be no. HU increases blood pressure and/or flow in the carotid artery and thoracic aorta (50, 59) and increases both eNOS and iNOS in these vessels. However, HU had little or no effect on blood flow in the mesenteric vasculature (33, 50), but decreased eNOS and iNOS in this vascular bed. HU decreases blood flow in the femoral artery but had no effect on either eNOS or iNOS. This phenomenological analysis suggests that, in addition to the known effects of shear stress on eNOS, HU produces changes in eNOS and iNOS by mechanisms that are independent of flow and/or pressure.

It is also important to consider whether the changes in NOS and NOx reported in the present study contribute to the previously reported decreases in vasoconstrictor responsiveness. In the case of the carotid artery, the answer appears to be yes. In our previous study (48), evidence was presented suggesting that HU decreased the carotid artery contraction to NE, in part, by increasing eNOS and iNOS function. The present finding that the expression of both of these NOS isoforms is increased in carotid artery, supports the role of NOS in mediating the HU effect. Because both eNOS and iNOS were increased in the thoracic aorta, it is at least theoretically possible that NOS contributes to the HU effect on the contraction (10) of this vessel. NO may also play the role in the effect of HU on the cerebrovasculature. HU causes an increase in myogenic tone in cerebral vessels mediated, in part, by a reduced role for NOS (15). The HU middle cerebral artery was found to be desensitized to a NO donor (14). Thus the present finding that NOx was increased in the cerebrovasculature raises the possibility that elevated endogenous NO was responsible for the desensitization. The role for NOS in the HU effect on contraction of femoral artery cannot be determined because of the discrepancy between present results and our laboratory's earlier study (48) as discussed above. However, NOS does not appear to contribute to the effect of HU in the mesenteric vasculature. Because HU reduced both NOS and NOx in this vascular bed, the HU-induced reduction in vasoconstrictor responsiveness (27) and myogenic tone (25) appears to have occurred, not because of, but despite, NOS mechanisms.

Zhang (61) has reviewed the evidence that the localized hemodynamic effects of HU produce differential functional and structural changes in arteries. However, the present results suggest that factors independent of hemodynamic shifts are also important in the regulation of vascular eNOS and iNOS expression and activity.

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